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Understanding the role of alkaline phosphatase as a possible marker for the evaluation of antiparasitic agents

Gustavo A. Martínez[1*], Luis E. Trujillo[2]

[1]Center of Nanoscience and Nanotechnology CENCINAT, Universidad de las Fuerzas Armadas – ESPE, Sangolquí, Ecuador.
[2]Life Science Department. Industrial Biotechnology and bioproducts research group. CENCINAT. Universidad de las Fuerzas Armadas – ESPE. Sangolquí, Ecuador.

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ABSTRACT

The release of alkaline phosphatase (AP) into media provides an enzymatic method for the evaluation of the effectiveness of antiparasitic drugs *in vitro*. However, to date, it has only been applied sporadically in some cestodes and trematodes. In this paper, we describe for the first time a cost-effective evaluation method to accurately determine larvae immobility or mortality after salt or starvation stress treatment in order to measure the released parasitic AP as an enzymatic stress marker. Our method avoids tedious and time-consuming visual counting of the model organism, human parasite nematode *Strongyloides venezuelensis* larvae. In opposition to previous reports, we found a linear relationship between larval immobilization due to stress treatment and the released AP from L3 larvae. This method could be applied to a preclinical evaluation of active antiparasitic compounds in AP producing nematodes and other parasites.

INTRODUCTION

Alkaline phosphatase (AP) has been frequently used for many years as a reference enzyme for both clinical and molecular approaches (Coleman, 1992). Its application in cestodes and trematodes is limited, primarily based in the application of AP quantification to evaluate mortality and new antiparasitic drugs (Swargiary *et al.*, 2013; Adnani Sadati *et al.*, 2016; Paredes *et al.*, 2013). Nevertheless, in published reports, there are some contradictions about AP release by different parasites.

Histochemical studies of nematodes (Anya, 1966; Sood and Kalra, 1977; Sayers, *et al.*, 1984) found AP in different locations, but no reports relate the presence of AP in the nematodes' cuticle with experimental evidence of enzyme secretion to the media during parasite starvation or under other stress conditions.

In 2008, Ruano (Ruano, 2008) worked with the nematode *Strongyloides venezuelensis* and used AP for the first time as a marker in a nematode to evaluate the relationship between mortality and enzyme activity. The results of Ruano, apparently contradict results reported by Stettler *et al*. (Stettler *et al.*, 2001) that related parasite death with AP concentration released to the supernatant. Stettler was the pioneer in the use of AP to quantify metacestode damage using *Echinococcus multilocularis* as a model. She found high AP activity during the 14 days of the experiment.

Additionally, Ruano (Ruano, 2008) evaluated the behavior of AP in the supernatant when different nitric oxide modulators were used and in all cases, abrupt changes in the AP concentration were observed. This indicates a close relationship between chemical dose concentration and AP release. Although these experiments showed very interesting results, the nematodes were always destroyed. This is a disadvantage of this practice if it is to be used as a suitable evaluation method to accurately determine larvae mortality or immobility after chemical or starvation treatments.

In this article, we show evidence of AP secretion in nematodes without cuticle destruction after parasite starvation and sodium chloride treatment. Our primary finding is that AP might be used as an immobility or mortality indicator in several

*Corresponding Author
Gustavo A. Martínez, Center of Nanoscience and Nanotechnology CENCINAT, Universidad de las Fuerzas Armadas – ESPE, Sangolquí, Ecuador. E-mail: guardian100303 @ yahoo.es

parasites, solving some apparently contradictory results described by other authors regarding the differences in AP behavior in different parasite species. Additionally, knowing the common behavior of this enzyme in different parasites, it is possible to predict its behavior and evaluate new antiparasitic drugs *in vitro* in a more efficient way.

MATERIALS AND METHODS

In vitro culture of *S. venezuelensis*

S. venezuelensis eggs were collected from subcutaneously infected Wistar rats five days after infection, the feces were cultivated with vermiculite for another five days, and finally, larvae were recovered with the Baermann method. The larvae were washed with a diluted solution of sodium hypochlorite and then with distilled water. The larvae counting was done using an inverted microscope. In a culture plate with 24 wells, 100 larvae were added for each 0.5 mL of distilled water and were counted using the microscope. Following larvae counting, the plate was incubated for 24 hours with light at 28 to 31°C. After the incubation time, AP activity in the supernatant was determined using the method described below.

Measuring alkaline phosphatase activity

AP released by *S. venezuelensis* was determined using the colorimetric AP detection system (alkaline phosphatase BR Cromatest® Linear Chemicals, Spain) and adapted to a microassay format in our lab. In detail, 5 µL of culture supernatant was added to 125 µL of reagent, mixed according to the manufacturer's instructions, and incubated for 24 hours at 37°C in the dark. In a 96-well plate, the optical density (OD) at 405 nm was read in an ELISA reader (Linear GEA, Spain). Distilled water without parasite incubation served as a blank. Results were expressed as corrected OD using the following formulas:

N° corrected larvae = highest number of total larvae in the experiment

real relation = (N° experimental immobile larvae)/(N° experimental mobile larvae)

N° immobile larvae real relation = real relation * (N° exp. immobile larvae + N° exp. mobile larvae)

N° corrected immobile larvae = (N° immobile larvae real relation * N° corrected larvae)/(N° exp. immobile larvae + N°exp.mobile larvae)

corrected OD = (ODsample * N° corrected larvae)/(N° corrected immobile larvae)

RESULTS AND DISCUSSIONS

The relationship between released AP activity and larval immobility is shown in Figure 1. In this first trial, 24 well plates containing 100 L3 larvae of *S. venezuelensis* per well and distilled water as a medium were used, as described in the methods. The main objective of this experiment was to cause the parasite death by starvation and to observe the release of AP into the media over time. However, a negative linear slope was obtained, indicating an increase in larval immobility but no increase in secreted AP production (Fig. 1, Table 1, and Table 2). Strikingly, Paredes *et al*. reported an opposite behavior for AP (Paredes *et al.*, 2013; Mahanty *et al.*, 2011) and the AP behavior found in the study carried out by Stettler *et al*. (Stettler *et al.*, 2001). Although different parasites were used in those experiments, we believe that they provide clues to understanding AP behavior under the same hypothesis that include our experimental results in the present study.

Table 1: Calculated values of corrected OD. The experiment was done with approximately 200 larvae per well.

Corrected OD	% larval immobility	OD sample exp	N° corrected immobile larvae	N° immobile larvae real relation	Real relation	N° corrected larvae	N° exp. Immobile larvae	N° exp. mobile larvae	N° exp. total	OD sample
2.199	54.032	1.982	122.653	103.202	0.540	227	67	124	191	1.189
1.712	54.032	1.719	122.653	103.202	0.540	227	67	124	191	0.926
3.714	30.459	1.925	69.144	69.144	0.304	227	53	174	227	1.132
3.356	30.459	1.816	69.144	69.144	0.304	227	53	174	227	1.023
3.663	31.788	1.958	72.159	63.258	0.317	227	48	151	199	1.165
3.937	31.788	2.045	72.159	63.258	0.317	227	48	151	199	1.252
3.369	24.852	1.631	56.414	52.438	0.248	227	42	169	211	0.838
3.446	24.852	1.650	56.414	52.438	0.248	227	42	169	211	0.857
2.998	36.538	1.889	82.942	77.827	0.365	227	57	156	213	1.096
2.932	36.538	1.865	82.942	77.827	0.365	227	57	156	213	1.072
0.601	100.000	1.394	227.000	190.000	-	227	190	0	190	0.601
0.323	100.000	1.116	227.000	190.000	-	227	190	0	190	0.323
0.870	Blank	0.870	-	-	-	-	-	-	0	-
0.717	Blank	0.717	-	-	-	-	-	-	0	-

Experimental data were taken with: 1.-24 hours of parasitic incubation. 2.-24 hours of analytical incubation.

Paredes *et al*. and Stettler *et al*. used different experimentation periods, 3 to 4 days and 14 days, respectively. In the case of Stettler *et al*., the AP increment in the media is by physical destruction of the parasite, a phenomenon that cannot

be observed within a few days of exposition, as in the case of Paredes *et al*. This explains the fact that, unlike in the Stettler *et al*. work, Paredes *et al*. reported no cuticle rupture, similar to what occurred in our experiment (Fig. 3). Therefore, in both our work and that of Parades *et al*., there is no measurement of enzyme release at the moment of *S. venezuelensis* death. However, with parasite destruction as described by Stettler, it is possible that the AP located inside the parasite was released in an untidy way.

Table 2: Calculated values of corrected OD. The experiment was done with approximately 100 larvae per well.

Corrected OD	% larval immobility	OD sample exp	N° corrected immobile larvae	N° immobile larvae real relation	Real relation	N° corrected larvae	N° exp. Immobile larvae	N° exp. mobile larvae	N° exp. total	OD sample
1.076	65.517	0.921	81.897	62.897	0.655	125	38	58	96	0.705
0.945	65.517	0.835	81.897	62.897	0.655	125	38	58	96	0.619
1.140	64.407	0.95	80.508	62.475	0.644	125	38	59	97	0.734
1.030	64.407	0.879	80.508	62.475	0.644	125	38	59	97	0.663
1.229	53.333	0.871	66.667	49.067	0.533	125	32	60	92	0.655
0.994	53.333	0.746	66.667	49.067	0.533	125	32	60	92	0.530
1.420	46.341	0.874	57.927	55.610	0.463	125	38	82	120	0.658
1.157	46.341	0.752	57.927	55.610	0.463	125	38	82	120	0.536
1.484	43.678	0.864	54.598	54.598	0.436	125	38	87	125	0.648
1.232	43.678	0.754	54.598	54.598	0.436	125	38	87	125	0.538
0.218	100.000	0.496	125.000	58.000	-	125	58	0	58	0.218
0.280	100.000	0.434	125.000	58.000	-	125	58	0	58	0.280
0.229	Blank	0.229	-	-	-	-	-	-	0	-
0.202	Blank	0.202	-	-	-	-	-	-	0	-

Experimental data were taken with: 1.-24 hours of parasitic incubation. 2.-24 hours of analytical incubation.

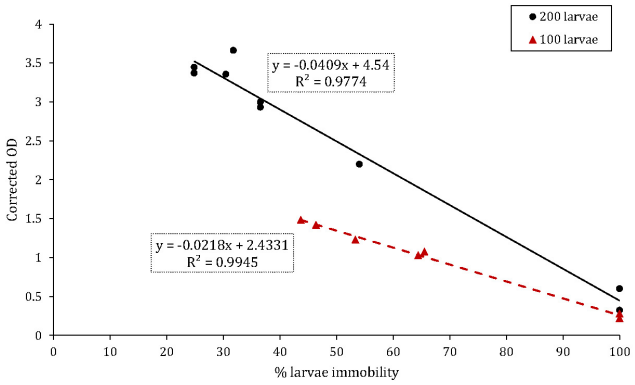

Fig. 1: Linear relationship between the percentage of immobile larvae versus absorbance at 405 nm in distilled water with 100 and 200 larvae.

Sayers *et al*. (Sayers, *et al*., 1984) carried out biochemical work on the surface of the nematode *Brugia pahangi*, finding AP in the cuticle, specifically the hypodermis, cortical, and basal structures, and suggested that there are active transport mechanisms in the cuticle. In 1995, Martinez and De-Souza (Martinez and De-Souza, 1995) made a complete study of the cuticle of *S. venezuelensis* and the similarities in the cuticles are notable, showing the same distribution of sheath, epicuticle, outer cortical, inner cortical, basal zone, and hypodermis. With this evidence, we could infer that *S. venezuelensis* must also have AP in their cuticle and that the behavior is the same in cestodes when this parasite is under starvation conditions.

Our results with 100 larvae of the same stage per well were replicated using 200 larvae under the same conditions and an identical trend was observed, thus verifying the accuracy of the results obtained in the first experiment (Fig. 1). These experiments were repeated with 100 and 200 larvae per well using a one molar saline solution instead of distilled water. We observed a logarithmic death that conserved a close linear relationship between AP production, AP release, and parasite death (Fig. 2). It is important to note that, as expected, the intercept at the y-axis (Fig. 2) of the lines for 200 and 100 larvae show that the amount of AP secreted by 200 live larvae is double the amount secreted by 100 live larvae.

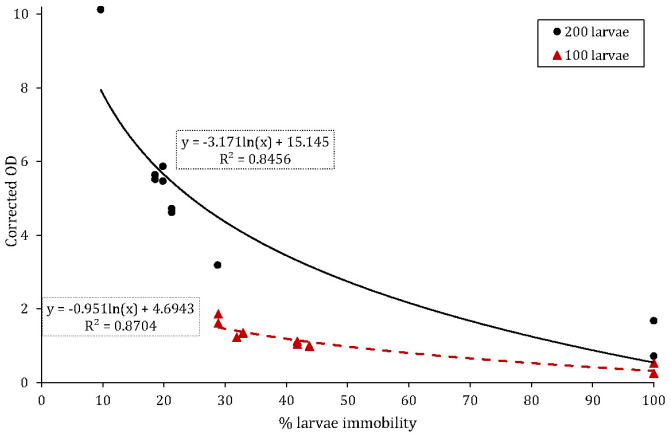

Fig. 2: Lose of linearity when the experiment is done in commercial saline solution, the activity of AP change. Approximately 100 larvae.

The observation that *S. venezuelensis* releases cuticle AP while it is alive demonstrates that other nematodes could have AP in their cuticles and release it following treatment with a test substance, but without destroying the parasite. If cestode walls or nematode cuticles are responsible for AP levels in the parasites

media, then we consider that should exist reports of histochemical studies where it is common to find AP in walls and cuticles. The result of searching nematodes' cuticle constituents gives us a list of nematodes with the similar histopathological constitution to *Brugia pahangi*, which is known to have AP in its cuticle (Table 3).

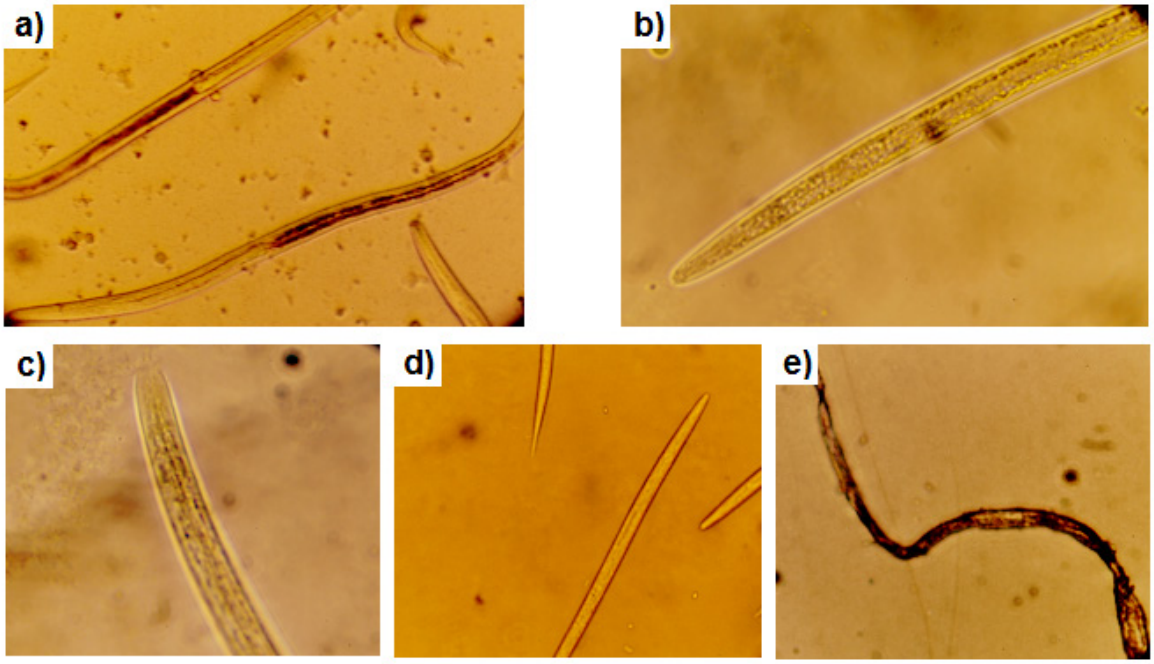

Fig. 3: Experimental photos of larvae. a) *S. venezuelensis* L3 larvae alive in the water, b) dead larvae in the pure reagent, few days permanency, c) larvae dead in the pure water, many days permanency (rupture), d) dead larvae in the pure reagent, few days permanency, e) larvae in NaCl 1M after a few hours.

Table 3: Literature reports of AP in nematodes' cuticle.

Nematode	Cuticle constituents reported	Reference
Strongyloides venezuelensis	Sheath (Surface coat), epicuticle, cortical layer, medial layer, fibrous layer, basal layer and hypodermis.	(Martinez and De-Souza, 1995)
Caenorhabditis elegans	Epicuticle, cortical, medial, fibre, basal and hypodermis.	(Page *et al.*, 2014)
Strongyloides ratti	Surface coat, epicuticle, amorphous layer (cortical layer), striated layer (fibrous layer), basal layer and hypodermis.	(Grove *et al.*, 1987)
Ascaris suum	Epicuticle, cortical zone, median zone, basal zone, basement membrane.	(Bird, 1991)
Haemonchus contortus	Cortical, matrix, fibre, hypodermis.	(Sood and Kalra, 1977)
Euchromadora vulgaris	External, second layer (composed of 2 sub-layers), third layer (composed of overlapping plates), basal layer, epidermis (the actually known hypodermis).	(Watson, 1965)
Ascaris lumbricoides	External cortical layer, internal cortical layer, fibrillar layer, homogeneous layer, fibre layers, basal lamella.	(Bird and Deutsch, 1957)
Nippostrongylus brasiliensis	Cortex, fibrils of collagen, strut or skeletal rod, fluid-filled layer of cuticle, fibre layer of cuticle, basement lamella.	(Lee, 1965)

Experiments carried out by Horiuchi *et al*. (Horiuchi *et al.*, 1959) demonstrated that AP in *Escherichia coli* had a behavior similar to that observed in this research, although there is a large difference between a bacterium and a nematode. *E. coli* release AP, though no phosphate is present in the media and AP secretion stops when phosphate is added. Therefore, this enzyme is regulated under stress as a consequence of phosphate presence or absence.

Mahanty *et al*. (Mahanty *et al.*, 2011) reported the same behavior observed by Ruano in controls, but completely opposite behavior when they used a drug compared to Ruano's use of active compounds. This cannot be explained if we consider that only dead parasites secrete AP.

Our next step was to mathematically simulate Ruano's results in terms of larval behavior and phosphatase production obtained in our experiments. The result was strikingly close because we were able to reproduce Ruano's slope of the curve and the variation coefficient accurately (Fig. 4). We could not, however, do a strict simulation because the rate of immobility is different in both experiments and we had to assume a rate for Ruano's experiments. Therefore, there is not sufficient data to reach a final conclusion.

We were then in the position to postulate a mathematical model of the process and its possible application in the daily analysis.

Mathematical simulation of AP release by *S. venezuelensis* larvae

The behavior of *S. venezuelensis*, as well as other parasites, agree with the equations that are deduced here.

The larval immobility (or mortality) follows the equation:

$$\% \text{ immobility} = A * e^{Bt} + C \qquad (1)$$

where t is time units.

Our findings show that optical density (OD) follows the equation:

$$\text{corrected OD} = -D * \%\text{immobility} + E \quad (2)$$

Replacing (1) in (2):

$$\text{corrected OD} = -D * (A * e^{Bt} + C) + E \quad (3)$$

Equation three shows the real relationship between enzyme secretion and time in a non-accumulative way. This is the OD that we should be able to read if we change the supernatant frequently and control the exact number of parasites in the analysis.

Knowing that Equation three describes the slope of each point of the curve, we were able to integrate it to achieve the accumulated OD:

$$(d \text{ accumulated OD})/dt = \text{corrected OD}$$

$$(d \text{ accumulated OD})/dt = -D * (A * e^{Bt} + C) + E$$

$$d \text{ accumulated OD} = (-D * A * e^{Bt} - DC + E) * dt$$

Integrating:

$$\text{accumulated OD} = \int(-D * A * e^{Bt} - DC + E)dt$$

$$\text{accumulated OD} = -D * A \int e^{Bt}\, dt - DC \int dt + E \int dt$$

$$\text{accumulated OD} = -D * A * B * e^{Bt} - DC * t + E * t + \varphi$$

To calculate the constant φ, we know the condition that at t = 0 the accumulated OD = $OD_{initial}$ and hence the final equation is:

$$\text{accumulated OD} = -ABD * e^{Bt} + (E - DC) * t + ABD + OD_{initial} \quad (4)$$

With this equation, it is possible to explain why some parasites have increased AP release following drug treatment when compared to controls, while decreased AP release is observed in other cases. It is important to mention that if we calculated the limits of Equation four when time trends to 0, we obtain $OD_{initial}$ as a logical result. For *S. venezuelensis*, it is possible to approximate the immobility equation related to natural death versus accumulated OD to a line, as we can verify in Equation four where the term $-ABD*e^{Bt}$ tends to be zero.

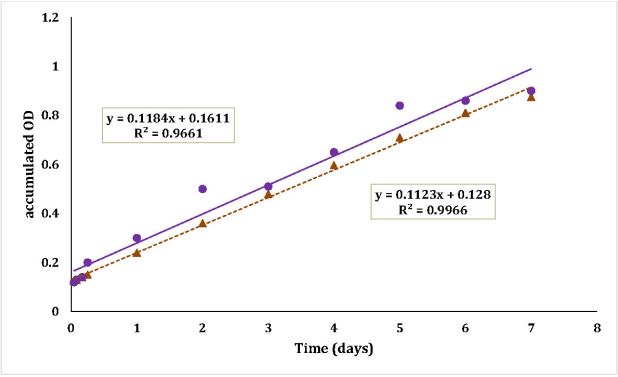

Fig. 4: Alkaline Phosphatase accumulated in the supernatant of Ruano's experiment (line) and the theoretical simulation (dots).

With these considerations, it was possible to simulate a curve of the AP accumulation in the media according to Ruano's experiment, considering some experimental data for that assay with some assumptions that our hypotheses encompass (Fig. 4). We use Ruano's experimental data that at seven days the percentage of immobility is 20%, this relation corresponds to the equation % immobility = 0.0119 * EXP(1.0619 * days). With the observations from our work, we know that the scope of the corrected OD versus the percentage of immobility must be negative and we arbitrarily chose the equation corrected OD = -0.004 * %immobility + 0.1. Applying Equation four, we obtain what could be an approximately linear relationship with a similar slope (fig. 4). This demonstrated that it is possible to achieve an AP increment in the media even though there are fewer larvae alive.

From a mathematical model to a useful experiment

The observation that the OD response (AP response) in the supernatant of parasites versus time is linear, constitutes an easy and powerful tool to evaluate the effects of drugs activity, active compounds from plants, or plant extracts in parasites. Since the action of active compounds agrees with the Michaelis-Menten response, we expect a similar response in accumulated OD in this kind of experiment. For example, when using a drug in this kind of experiment, the parasites begin to die in a logarithmic way and knowing that the response of accumulated OD is almost linear with respect to time, we expect a Michaelis-Menten response to the drug in terms of accumulated OD.

It is necessary to mention that for routine experiments we would only need to calculate the accumulated OD, as calculation of the corrected OD was needed only for the purposes of this article.

With all of these considerations, we are now able to propose a general method to evaluate the half maximal inhibitory concentration (IC_{50}) of compounds utilizing AP activity as an indicator of inhibition. The following is a protocol example using microscopic nematodes.

Collect and wash larvae then place them in distilled water. Using a micropipette, transfer a drop of known volume to a microscope and determine the larvae number. Then calculate the volume needed for each well according to the number of parasites desired for the experiment. Each well contains a volume of distilled water with and without different drug doses. Controls should show a linear response of OD versus time, and the drug effect should produce different Michaelis-Menten kind of responses in the same graph. Finally, plot the accumulated OD versus drug concentration (To calculate the IC_{50}, choose different concentrations of the drug but the same treatment time during the experiment) to obtain a Michaelis-Menten curve. The accumulated OD = J * Dose/(K + Dose) where the IC_{50} is calculated with the equation:

$$\frac{1}{\textit{accumulated OD}} = \frac{K}{J}\left(\frac{1}{\textit{Dose}}\right) + \frac{1}{J} \quad (5)$$

and

$$IC_{50} = K$$

The main advantages of this new approach are that we do not have to count each parasite using a microscope and we can use hundreds of parasites without problems. Indeed, it is better

to use larger numbers of parasites because we achieve increased AP secretion. This resulted in a faster response and negligible OD corrections.

CONCLUSIONS

AP activity has been widely used to evaluate new drugs *in vitro*, but previously published results report some contradictory information about AP release by different parasites. We report an experimental approach using AP activity in the media as an immobility or mortality indicator in several parasites. The use of this approach solves some of the contradictory results described by other authors regarding the AP behavior in different parasites species.

From the results obtained, it is possible to propose a methodology for the evaluation of potentially active compounds *in vitro*. This process is easier because it avoids a majority of the classical process for microscopic larval counting. Additionally, we propose that AP activity in the media may be used as a death marker, providing that the parasite corpses do not undergo physical damage to their structure. In our experiments, when the AP activity of a group of parasites begins to decline it indicates that they are dying.

Knowing the common behavior of this enzyme in different parasites, it is possible to predict its behavior and evaluate new drugs *in vitro* in a more efficient and cost effective way. Also, this research solves some of the apparently contradictory results described by other authors regarding the AP behavior in different parasites species.

A mathematical model was proposed to explain the behavior of AP release observed in *S. venezuelensis*. This model is applicable to many parasites and is the fundamental basis for the postulation of a formula for the calculation of the IC_{50} using AP as a parameter to be measured in some parasites.

The release of AP into starvation media by cestodes, nematodes, and bacteria could be due to a natural response of the organism in search of nutrients to be absorbed by the cuticle or cell wall. This stress phenomena is not only seen in the absence of nutrients, but also during the alteration of multiple variables that may stress a parasite, including temperature, pH, and drug exposure.

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